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DEVELOPMENT OF A UNIVERSAL ASSAY FOR MEASURING IMMUNOGLOBULIN LEVELS IN CATTLE AND GAME-FARMED ANIMALS

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Development of a Universal Assay for Measuring Immunoglobulin Levels in Cattle and Game Framed Livestock

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Abstract

Immunoglobulins are proteins produced by B-lymphocytes; these molecules are commonly referred to as antibodies. Immunoglobulins are important components of the immune response, capable of binding to viruses, bacteria and other pathogens and assisting in the recovery of the animal following infections. The health of most newborn domestic animals is dependent upon receiving adequate levels of immunoglobulins from the mother by suckling of the first milk or colostrum. The successful transfer of maternal immunoglobulins, via the colostrum, can only be assessed by testing the blood of the offspring after suckling to ensure that adequate levels, particularly of immunoglobulin class G (IgG), have been achieved. The assay traditionally used to perform this test is a radial immunodiffusion (RID) method. The RID method, while accurate, requires expert technical skills, and is expensive and time-consuming. Enzyme-linked immunosorbent assays (ELISA) is an alternative technical method, which has become the assay of choice for most rapid diagnostic techniques. However, only for horses is there a commercially available rapid ELISA for measurement of serum immunoglobulins. The goals of this study are to develop ELISA-based test methods for use in other domestic animals, particularly in cattle and game-ranched animals.

The first goal of the study was the development of a conventional ELISA method for use in cattle. The first hurdle overcome to achieve this goal was the assessment of the commercially available reagents for bovine immunoglobulins to determine which would provide the most accurate assessment of the entire population of bovine IgG. Ten commercially available antisera to bovine IgG have been evaluated in RID methodology using standard serums and purified bovine IgG from 11 sources. The results of these assessments have shown that some of the antisera fail to detect the entire population of serum IgG and conversely that some of the standards sold commercially contain only one of the subclasses (either IgG 1 or 2). These studies have been used to select antisera and standards for optimization of the RID and development of an ELISA method. The conventional ELISA, using reagents broadly reactive to bovine IgG has been developed and optimized. The next goal of the study was to substitute molecules capable of binding to the immunoglobulin of animals other than cattle to produce a universal ELISA, which could be used for other species in addition to cattle. Thiophilic polymers and Staphlococcal proteins have been tested for this purpose. One of the Staph proteins, Staph protein G was shown to highly effective when substituted for the bovine specific antisera in the conventional ELISA. This protein was also been shown to bind to the IgG molecules of some of the game -farmed animals.

The majority of the work since the last progress report has been in the development and validation of conventional and "universal" ELISA based assays for evaluation of IgG of bison, alpaca and llamas, elk and deer. Using reagents supplied by colleagues at the CFIA we have validated and established conventional ELISA methods which can be available to the Saskatchewan agricultural community through the Prairie Diagnostic Service. We have also worked to develop the prototypes of the "universal" ELISAs , substituting the Staph. Protein G for the species specific reagent to immunoglobulin. In all the target species this was shown to be an effective approach and could be substituted for the species specific reagents which has advantages in terms of availability and cost savings.

The final goal of the study was the development of a prototype "chromatography-based" rapid assay for cattle and other species. The prototype showed proof of principle in cattle, however an assay based on similar technology became commercially available for cattle in the USA through a company called Midland BioProducts Corp. Our tests of this assay have shown that it is of good quality and we have suggested technical improvements to the current kit that will improve its accuracy. Our initial tests show that the assay, while perhaps not "universal" to all game-farmed species may have application to some species other than cattle. The follow up plans for project is now to continue to collaborate with Midland BioProducts Corp. to further test and apply the rapid kit for cattle and to develop the commercial kits for application to game-farmed species.

Executive Summary

The highest numbers of death losses in cattle and other farmed veterinary species is from infectious disease in the first 30 days of life. Many studies have documented that the risk of infectious disease in this time period is directly related to the levels of serum antibodies (immunoglobulins) in the neonate. These immunoglobulins are obtained by absorption from the mother's colostrum. The measurement of serum immunoglobulins is the only reliable means to identify neonates that have received inadequate colostral immunoglobulins and thus are likely to have disease and/or death losses. Knowledge of the neonates immunoglobulin status can be used in making decisions regarding the prognosis and treatment of individual animals and in providing suggestions for herd husbandry and management practices.

The test currently considered the gold standard for measurement of serum immunoglobulin is the radial immunodiffusion (RID) method. The RID is a precipitation-based assay in which the immunoglobulin in the patients' serum is precipitated in an agar gel by the incorporation of species-specific antisera against immunoglobulin. The RID can be accurate and highly reproducible however it is slow (requiring over 24 hours to develop). RID assays are also very expensive, as very high levels of antisera are required for incorporation in the gel. An additional limitation of the RID is that since the assay is technically demanding it cannot be performed by personnel other than those highly skilled in laboratory techniques. There is a need for an assay that is more rapid, less expensive, and less technically demanding; both for use in the laboratory and ultimately for field use.

In recent years farming has diversified from traditional livestock such as cattle to include the rearing of many additional species, including bison, deer, alpaca, and llamas, some of which currently have large individual worth. While there are commercially available reagents for use in RID assays for cattle and recently for llamas there are no reagents commercially available for use in the other economically important species. There is a need for IgG assay methods applicable to these animals. It is a goal of this project to develop an assay, which can rapidly and inexpensively measure immunoglobulin levels in game-farmed animals as well as in cattle.

One method that has replaced many other types of serological test methods is the enzyme linked immunosorbent assay (ELISA). This method has advantages over precipitation based methods such as RID in terms of speed, economy of reagents and numbers of samples that can be simultaneously tested. ELISAs can be designed to be robust and many of the shelf-stable diagnostic kits used by veterinarians in the field or clinic are ELISA technology. The only kit commercially available for rapid measurement of immunoglobulin in veterinary species when this project was initiated was an ELISA-based kit used in neonatal horses. There were no rapid ELISAs available for other species including, surprisingly, cattle, which have been raised in large numbers and have been of significant economic importance for a long time.

In addition, while there are many manufacturers which market commercially available reagents for measurement of IgG in cattle, it is apparent that there are significant discrepancies among the results obtained with reagents from different manufacturers when these are used in the conventional RID method. One of the goals of this study was to evaluate the commercially available reagents for measuring IgG in cattle and to determine which reagents most accurately reflected the levels of IgG present. Based on these studies recommendations can now be made on the use of standards for both conventional RID methods and for the development of new tests. The next objective was to develop, using the reagents shown to be most accurate, an ELISA-based method. The goal was to develop an ELISA that could be used, in the first instance, for laboratory-based assessment of bovine immunoglobulins and secondly, would form the foundation for the development of a rapid assay, which could be used in the veterinary clinic or on the farm.

An additional goal of the study is to develop broadly useful assay methods, which are applicable to game-farmed species in addition to cattle. Generally, the reagents used in RID assays or ELISA for the measurement of immunoglobulin are antisera raised in rabbits or other mammals by immunization with immunoglobulin of the target species. These antisera are therefore primarily directed against the immunoglobulin of the immunizing species and will be only partially cross-reactive or unreactive in recognition of the immunoglobulins of other species. Thus antisera raised against bovine immunoglobulin may be partially but not completely cross-reactive to that of related species such as bison and may fail entirely to recognize the immunoglobulins of unrelated species such as alpacas. An alternative to using antisera to immunoglobulins is the use of other agents with binding affinity for IgG. An example is bacterial protein; there are two specific proteins, (Staph protein A and Staph protein G) found in Staphylococcal bacteria. These proteins have high binding affinity for the immunoglobulins of many species, are commercially available in highly purified forms, and have proven efficacy in other types of immunoglobulin binding assays such as immunohistochemistry. There are also synthetic immunoglobulin-binding molecules commercially available; these are thiophilic polymers (T-gels) which are designed to bind to a highly conserved portion of the immunoglobulin molecule. T-gel has been used primarily for the purification of monoclonal antibodies (mouse immunoglobulin) however its universal immunoglobulin binding capacity may enable it to replace species specific antisera in other types of assays in other species as well. An objective of this study is to examine the potential of the immunoglobulin-binding Staph. proteins and/or the thiophilic polymers in the development of a universal assay to measure the immunoglobulin levels in game-farmed animals as well as in cattle.

The first objective of the study was the assessment of the commercially available reagents for evaluation of cattle immunoglobulins. These reagents include antisera (usually of rabbit or sheep origin) and standards with "known' amounts of IgG or IgG1 and/or IgG2. There are no reagents sold commercially specifically for evaluation of IgG3 in cattle and it is unclear as to the contribution of this IgG subclass to total IgG. The results of the assessments of the bovine IgG standard in our studies have demonstrated significant variability among the commercial standards. Some standards were shown to be primarily IgG1 while others are primarily IgG2 and others serum with "normal" adult bovine proportions of IgG1 and IgG2. We have identified 2 bovine standards, one from Bethyl Laboratories and one from Jackson Laboratories, which

have normal proportions of IgG1 and IgG2 and appear to contain levels of IgG within 10-15% of the manufacturer's claims.

We have also tested antisera from 8 commercial sources in RID assays for bovine IgG. Based on our evaluations, the assessments for bovine IgG should be conducted with reagents which are broadly crossreactive to all subclasses; these reagent are those which are directed to IgG whole molecule. Reactivity to whole molecule implies reactivity against the light chain as well as the heavy chain of the immunoglobulin (each IgG is comprised of 2 gamma or heavy chains and two light chains). Antisera directed to the light chains which have not been shown to have animal to animal variability in structure ensures that minor animal to animal variations in the structures of the heavy chains will not result in an underestimation of the amounts of IgG present. We have identified several antisera, including those from Bethyl, Jackson, Sigma or ICN Laboratories, which are suitable for bovine IgG testing.

The RID method currently offered at the Western College of Veterinary Medicine in the research laboratories has been revised to reflect the findings of our studies. Similarly the tests now offered through the Prairie Diagnostic Service to Saskatchewan producers now uses the standard serums and antisera and assay methods as developed by our studies. We have also reported the results of our studies and made these recommendations to the laboratory services of the Canadian Food Inspection Agency (CFIA) and United States Department of Agriculture (USDA) and presented our findings to other USDA, academic and industry representatives at a public meeting hosted by the USDA in Ames Iowa. The CFIA Veterinary Biologics Evaluation laboratory has adopted the protocol developed in our studies and the USDA will likely similarly approve this protocol within the next 1-2 months.

An effective conventional antigen capture ELISA method has been developed for determination of bovine IgG. Several antisera were evaluated using Bethyl Laboratories standard serum as the positive control and fetal calf serum (which contains no IgG) as the negative control. Many antisera were found to contain populations of antibodies binding to other non-immunoglobulin molecules in the bovine serums and/or to the immunoglobulin of the species in which the antisera was raised. An effective method was developed by using sheep-derived antisera from Bethyl Laboratories as both the capture and detection antisera. This method has many advantages over the RID method in terms of speed, technical ease, lower costs and ability to test larger numbers of specimens.

The next phase of this project was the replacement of the species-specific antisera in the conventional ELISA with molecules able to bind to IgG of other species in addition to cattle. Our studies found that the thiophilic polymers were unsuitable for this purpose. It appears that the thiophilic polymers have a single binding site for the IgG (thus are unable to cross-link the molecules) and in addition to binding to the IgG also binds to Staph proteins including Staph protein G. In contrast, one of the IgG-binding proteins of Staphylococcus (Staph Ptn G) was highly effective. This molecule, when substituted for the species specific sheep anti-bovine IgG, was much more potent (able to be used at much higher dilution) in testing for bovine IgG. The next phase of the study was the testing of a bank of serum from cattle of different ages to establish the normal ranges in the conventional ELISA assay. The results of these tests demonstrated that the

conventional and "universal" format ELISAs accurately detect the IgG in both neonates and mature cattle as well as in colostrum.

We have developed laboratory-based assays for immunoglobulin measurement in game-farmed animals. Given the absence of commercially available and validated immunoglobulin standards our approach was the development first of "gold standard" RID assays for each species, then, using the results of these tests to develop the ELISA based methods. We have commercially available reagents (both standards and antisera) for llama and alpacas and we used these reagents to standardize RID based assays. We developed a collaboration with Dr Deli Ogunremi at the CFIA in Saskatoon to pursue the development a test for elk, bison and deer, the game-farmed species with the greatest commercial value and of most concern to producers. We have reagents (both standards and antisera) supplied by Dr Ogunremi which we used to standardize a conventional RIDs. The RID tests for each species were then used as templates to adapt that test to a conventional ELISA (using species specific antisera). The ELISA method for each of the target species were established and validated and panels of serums from adults and neonates (when available) used to develop normal ranges.

The next phase of the study was to determine whether the species specific antisera used in each individual assay could be replaced with the Staph Protein G as a universal detection agent for the game-farmed species. We had tested, previously, the Staph. Protein G binding for the main species of interest in game-farming and the results suggested that the reagent would be applicable to the tested species. Since the last progress report, the Staph protein G conjugate was substituted for the species specific antisera and an ELISA method was standardized for each of the target species. Thus it was shown that a universal detection system can be employed for game farmed animals as well as for cattle. This has great advantages as the species specific reagents for the game farmed animals used in the present study are not commercially available (other than for alpaca and llama) and those which were supplied by the CFIA are of finite quantities. The use of the Staph G reagent ensures that the assays can be done even if these antisera are not available. In addition it suggests that an assay can be devised which could be applied (animal side) to all species. However, while it is desirable, it is not critical that the animal side assays be developed to be "universal." Many of the game farmed species are of sufficient economic value that it may be cost-effective to produce and license kits specifically designed for each species.

The development of a rapid assay suitable for use in the field was the final goal for this project. The major application for such an assay is for testing for failure of passive transfer of maternal immunoglobulin in calves. We have developed a protocol for an immuno-chromatographic prototype test using the Staph. Protein G reagents for cattle. The prototype results suggest that either the specific anti-bovine IgG reagents or the Staph Protein G could be impregnated on membranes and used to detect the IgG levels in serums of calves. The next stage, the development of test kits, would require a collaboration with a company with specific expertise in kit development and licensing. I have a quote from a US firm that specializes in such kit development that the initial kit and first phase testing could be completed for $40,000-$60,000 (US). It was our original plan, at this stage to seek a commercial partner as a rapid kit for cattle has significant commercial potential but would require large investments and expertise in "kit" development and licensing. In recent months such an assay has been released into the

market-place. It is an immuno-chromatographic method for determination of immunoglobulin in calves that gives a rapid and robust measure of passive transfer. The assay is marketed by Midland BioProducts Corp. (information appended). A patent for the technology has been applied for. We have tested the assay produced by this company and found that the results are very good (compared to the results found in our ELISA and RID values) and have been able to suggest technical changes to the method for further improvement in the results. We have had discussions with the scientists at Midland BioProducts Corp. and are pursuing the development of a collaboration to further develop their assay for calves and to develop joint projects for field tests to evaluate the kits' application. There is also continued interest at Midland BioProducts Corp. to develop a rapid assay for game-farmed animals

Technical Report

1. Identification of Optimal Reagents for Radial Immunodiffusion (RID) based IgG Measurement in Cattle:

To enable the development of reliable methods for determination of the levels of immunoglobulins in serum of cattle and other species, the first phase was the identification of reagents for accurate RID analysis of immunoglobulin in cattle. Two types of reagents are required, a standard with an accurately known level of immunoglobulin and an antisera capable of binding to and precipitating bovine immunoglobulin.

The standard is either a bovine serum or purified IgG preparation. The measurement of the reaction obtained with the standard is used as the guide for calculation of the relative levels in test samples. While many companies sell standards for measurement of bovine IgG is there no means to know if all (or any) are accurate, thus it was necessary prior to further test development, to attempt to determine which standards were most likely to be accurate.

The second reagent required for determination of serum immunoglobulin levels, the antisera, is a hyperimmune serum (usually of rabbit, sheep or goat origin) which is directed against bovine IgG. In the RID assay, the antisera are used to precipitate the immunoglobulin from the test sample to form the visible area of diffusion in the agar gel. It is important that the antisera contain populations of antibodies able to recognize all of the 3 known subclasses of bovine IgG and that the antibodies recognize the various allotypes of IgG (so that the IgGs of all individual animals will be recognized).

Twelve commercially available bovine IgG standards and 10 commercially available antisera to bovine IgG (Tables 1 & 2) have been tested in RID assays to identify those reagents which most reliably measure the IgG in bovine serums. An assumption was made that those standards that measured within 10%-15% of the manufacturer's claims using antisera from another manufacturer were most likely to be accurate. The RID tests were then performed using the standards and antisera supplied by each manufacturer to test for the IgG level in the bovine IgG standard (purified IgG or reference serum) of the other manufacturers.

The results were considered to show acceptable agreement among the tests and with the manufacturers claims if the assays measure within 10-15% of the manufacturers claims with heterologous antisera and controls as this is consistent with the expected accuracy of an RID test. Shown in bold type in Tables 3 & 4 and summarized in Table 5 are those assays which were within 10% of the manufacturers claims, those superscripted with * are those within 15% of the manufacturer's claims. In Table 3 the results of the two kits for measurement of IgG1 and IgG2 are added together for calculation of total IgG.

The details for the performance of the RID method are attached appendices.

Table 1. Bovine IgG Standards and Manufacturers Claims:

1. Bethyl Laboratories bovine serum standard: (IgG: 21.3 g/L)
2. VMRD bovine serum standard: (IgG: 16.5 mg/mL)
3. Sigma Chemical Co. bovine serum standard (IgG: 20 mg/mL)
4. ICN bovine serum standard (IgG: 20 mg/mL)
5. Jackson Laboratories whole molecule purified IgG standard (IgG: 27.6 mg/mL)
6. Jackson Laboratories IgG1 standard (10.8 mg/mL)
7. Jackson Laboratories IgG2 standard (22 mg/mL)
8. Chemicon Laboratories whole molecule purified IgG standard (20 mg/mL)
9. Chemicon Laboratories IgG1 standard (11.2 mg/mL)
10. Chemicon Laboratories IgG2 standard (22 mg/mL)
11. Biogenesis Laboratories bovine serum standard (IgG: 25 mg/L
12. NVSL bovine serum standard (10 mg/mL)

Table 2 Kits and Antiseras for determination of bovine IgG:

1. VMRD IgG (H) kit
2. Bethyl Laboratories IgG (H) kit antisera
3. Bethyl Laboratories IgG1 kit antisera
4. Bethyl Laboratories IgG2 kit antisera
5. Triple J Farms IgG kit antisera
6. Bethyl Laboratories IgG (whole molecule) antisera
7. Sigma IgG (whole molecule) antisera
8. ICN IgG (whole molecule) antisera
9. Jackson Laboratories IgG (whole molecule) antisera
10. Biogenesis IgG (whole molecule) antisera

Table 3. Results of RID tests of bovine IgG standards using 5 commercially produced kits for RID -based estimation of bovine IgG (H chain specific antisera).

	RID Reagents				
	VMRD (H) kit	Bethyl (H) kit	Bethyl IgG1 kit	Bethyl IgG2 kit	Triple J kit
IgG Standard					
Bethyl Std sera(-3) 21.3 mg/mL/	25*	22	10	9	24*
VMRD Std sera 16.5 mg/mL	16	16	16	2	12
Sigma IgG 20 mg/mL	24	24	0	20	18
ICN IgG 20 mg/mL	12	23*	6	15	21
Jackson IgG 26.7 mg/mL	18	27	17	12	28
Jackson IgG1 10.8 mg/mL	14	12	12	0	ND
Jackson IgG2 22 mg/mL	16	27	1	23	ND
Chemicon IgG1 11.2 mg/mL	13	10	10	0	ND
Chemicon IgG2 22 mg/mL	17	26	1	20	ND
Chemicon IgG 20 mg.mL	12	23*	15	7	ND
Biogenesis IgG 25 mg/mL	27	23	15	7	ND
Within 10%	2/11	6/11	10/11		3/5
Within 15%	3/11	8/11	10/11		4/5

Table 3 presents the results of examination of the 11 commercial standards with 4 commercially available pre-poured RID kits. From the data presented it appears that the kit from VMRD is the least accurate of those tested, measuring only 2/11 standards within 10% of the manufacturers claims and 3/11 to within 15% of manufacturers claims. In addition, this kit, in particular, cannot be recommended as it is too small to perform adequate numbers of replicates, the rings of precipitation are diffuse and difficult to measure, are often oblong rather than circular, and often overlap; all of which contribute to lack of accuracy and reproducibility.

The kits from Bethyl Laboratories and Triple J Farms which are IgG gamma chain (H chain specific) measured 6/11 and 3/5 (respectively) standards within 10% of the manufacturers claim and 8/11 and 4/5 to within 15% of manufacturers claims.

The kits from Bethyl Laboratories which are specific for IgG1 or IgG2, measured 10/11 of the standards accurately (when the measured levels were added); suggesting that if kits to the H chain of immunoglobulin are employed, that the most accurate measurements would be obtained using a combination of these two kits.

The results of testing the standards with the Bethyl kits, which distinguish bovine IgG1 from IgG2, also demonstrated that while the Bethyl and Jackson standards are balanced for IgG1 & 2, that the standards from VMRD and ICN are biased to one subclass. Thus for the purposes of this study and other studies in which it is desirable to measure IgG from a heterogenous group of cattle, the standards from Bethyl and Jackson Laboratories appear to be most suitable.

In addition to the other disadvantages discussed previously for RID assays, the commercially prepared kits have additional limitations in that the gels are small so only a few samples can be tested, they are expensive and have a limited shelf-life.

Table 4. Results of tests of bovine IgG standards using 5 commercially available IgG (H & L) antisera for RID -based estimation of bovine IgG in WCVM laboratory-prepared gels.

	RID H & L Antisera			
	Jackson H&L Jackson controls	Sigma H & L Sigma controls	ICN H &L ICN controls	Bethyl H &L Bethyl contr.
IgG Standard				
Bethyl Std sera 21.3 mg/mL	**22**	25*	25*	**22**
VMRD Std sera 16.5 mg/mL	14*	**15**	19*	13
Sigma IgG 20 mg/mL	**19**	**20**	27	**20**
ICN IgG 20 mg/mL	**18**	**21**	**20**	**19**
Jackson IgG 27.6 mg/mL	**27**	**26**	**25**	**26**
Jackson IgG1 10.8 mg/mL	13	**12**	13	14
Jackson IgG2 22 mg/mL	**21**	**21**	**21**	**23**
Chemicon IgG1 11.2 mg/mL	14	17	18	**13**
Chemicon IgG2 22 mg/mL	**21**	24*	**21**	19*
Chemicon IgG 20 mg/mL	25	28	29	23
Biogenesis IgG 25 mg/mL	15	17	14	13
Within 10% Within 15%	6/11 7/11	6/11 8/11	5/11 7/11	6/11 7/11

RID H & L Antisera

IgG Standard	Biogenesis H & L Biogenesis Stds
Bethyl Std sera 21.3 mg/mL-3	30
VMRD Std sera 16.5 mg/mL	19*
Sigma IgG 20 mg/mL	28
ICN IgG 20 mg/mL	na
Jackson IgG 26.7 mg/mL	41
Jackson IgG1 10.8 mg/mL	18
Jackson IgG2 22 mg/mL	40
Chemicon IgG1 11.2 mg/mL	na
Chemicon IgG2 22 mg/mL	na
Chemicon IgG 20 mg/mL	na
Biogenesis IgG 25 mg/mL	**22***
Within 10% Within 15%	1/8 2/8

Table 4 presents the results of testing the bovine IgG standards using antisera to bovine IgG incorporated into agar gels prepared in our laboratory. In laboratory prepared gels are much less expensive than the prepoured commercially available gels, and are larger so that more controls and replicates can be used.

The results of RID testing the IgG (H&L) antisera (and corresponding standards) from the 5 commercial sources also yielded some variability in results. However, the results obtained with the reagents from Jackson, Bethyl and Sigma Laboratories found over half of the standards tested to have values within 10% of the manufacturers claims while the ICN reagents found less than half the standards to be within 10% of the claim.

The majority of the standards measured within 15 % of manufacturers claims when using antisera from either Bethyl, Jackson, ICN or Sigma laboratories suggesting that any of these reagents could be used for this assay. The reagents from Biogenesis Labs measured only a minority of standards measuring within 10 or 15 % of the claim.

The data from all assays is summarized in Table 5. The summary of the RID testing shows that the whole IgG molecule standards from either Bethyl, VMRD, ICN or Jackson Laboratories, measures within 15% of the manufacturers claims in heterologous assays.

Since the standards from VMRD and ICN are biased to one subclass (Table 3), for the purposes of this study it is considered desirable to choose standards and antisera from Bethyl and Jackson Laboratories.

Table 5. Summary of the numbers of assays in which the standards measured to within 10% and within 15 % of the manufactures claims.

IgG Standard Claims	Assays Measured Within 10% or (15 %) of Manufactures		
	IgG (H) Kits	**IgG (H & L) Reagents**	**Total**
Bethyl Std sera 21.3 mg/mL-3	3/4 (4/4)	2/4 (4/4)	5/7 (8/8)
VMRD Std sera 16.5 mg/mL	3/4 (3/4)	1/3 (3/4)	4/7 (7/8)
Sigma IgG 20 mg/mL	2/4 (2/4)	3/4 (3/4)	5/8 (5/8)
ICN IgG 20 mg/mL	2/4 (3/4)	4/4 (4/4)	6/8 (7/8)
Jackson IgG 26.7 mg/mL	3/4 (3/4)	4/4 (4/4)	7/8 (7/8)
Jackson IgG1 10.8 mg/mL	2/3 (2/3)	3/4 (4/4)	5/7 (6/7)
Jackson IgG2 22 mg/mL	1/3 (1/3)	4/4 (4/4)	5/7 (5/7)
Chemicon IgG1 11.2 mg/mL	2/3 (2/3)	0/4 (0/4)	2/7 (2/7)
Chemicon IgG2 22 mg/mL	1/3 (1/3)	2/4 (4/4)	3/7 (5/7)
Chemicon IgG 20 mg/mL	0/4 (2/3)	0/4 (0/4)	0/8 (2/7)
Biogenesis IgG 25 mg/mL	3/3 (3/3)	0/4 (0/4)	3/7 (3/7)

2. Development of an Enzyme Linked Immunosorbent Assay for Bovine IgG.

The assay conditions for development of an enzyme-inked immunosorbent assay (ELISA) for bovine IgG have been established and validated.

Using the results obtained with the RID, the standard serums from Bethyl and Jackson Laboratories was selected as accurate and representative of normal bovine serum with respect to subclass distribution and were used in validation of the ELISA.

The ELISA requires two antisera to bovine IgG, rather than one as is needed for the RID method. The ELISA requires one antisera (coated onto the solid surface) which captures the bovine IgG and a second antisera (conjugated to the enzyme) which acts as the detection system.

We have tested a series of antiserums for ability to bind to the bovine immunoglobulin as both capture and detection reagents. A major limitation of the use of the two antisera is that they must bind to bovine immunoglobulin but must not bind to other bovine proteins or to immunoglobulins of the species in which the antisera was raised. For example, if the capture antiserum is raised in rabbits then the detection antisera must bind to bovine immunoglobulins but not to immunoglobulin of rabbits. Optimal results were found with sheep anti-bovine IgG (H &L) from Bethyl Laboratories used as the capture reagent and peroxidase conjugated sheep anti-bovine IgG (H &L) also from Bethyl Laboratories used as the detection reagent. All tests initially examined the binding to the IgG in the standard bovine serum from Bethyl Laboratories as the positive control and the binding of fetal bovine serum (which is IgG-free) as the negative control.

The optimal concentrations for each reagent are determined by testing each in a series of doubling dilutions in a "checkerboard' titration against the other reagent. The net values are optical density of the test sample (Bethyl standard serum or fetal calf serum) minus the value obtained in absence of the serum. The details for the standardized procedure for the ELISA is in the appendices.

The development of this ELISA provides significant advantages over the previously used RID method. The entire procedure can be completed in hours instead of over 2 days as is required for the RID, the reagents are used at much higher dilutions so the costs are significantly diminished (less than $1/10^{th}$) of the RID. In addition, over 40 samples can be tested/ELISA plate compared to 10 using our in-house RID and only 2-5 samples when using commercial pre-poured RID plates.

4. Development of a "Universal" ELISA for Cattle

The conventional "antigen capture" ELISA as outlined above for bovine IgG measurement relies upon two antisera specific to the IgG of the species being tested. While there are a variety of reagents commercially available for the IgG of cattle, there are few or no reagents for other species, particularly for the valuable game-farmed species. In order to develop an ELISA which is widely applicable to other species it will be necessary is to substitute other IgG-binding proteins for the species specific antisera in use in the conventional antigen capture ELISA. There is the need for replacement of both the IgG capture reagent and of the detection reagent.

Two types of immunoglobulin-binding molecules have been tested for their suitability in an ELISA for bovine IgG measurement. One of the molecules examined is the "T gels", thiophilic polymers, which have been used for the purification of monoclonal antibodies. The T gel has been purchased bound to small spheres; these spheres have been tested in microscopic assays for binding to bovine immunoglobulins. In contrast to antisera to bovine IgG the T gel coated spheres were not macroscopically or microscopically agglutinated by the bovine IgG implying that the T gel may not be able to cross-link bovine IgG. The ability to cross-link the IgG is important if the assay is to be moved into a rapid technology. Another limitation of the T gel is that it was found to bind to Staph protein A and G implying that these reagents could not be used together in an assay.

The other group of molecules which have been tested for IgG binding are the bacterial proteins, Staphlococcal proteins A and G. These are known to bind to the IgG molecules of some (but not all) species. We have systemically replaced the species-specific antisera to bovine IgG used as both capture and detection reagents with Staph proteins. Staph Protein G (but not protein A) was found to be more potent as both capture and detection reagents than the species-specific antisera. Cross titrations established that very high dilutions of reagents were effective in detection and quantification of bovine IgG. The method is described in the appendices..

To compare the binding of the Staph proteins to cattle and other target species for this study the serums of the various species were coated onto ELISA plates and the ability of enzyme-conjugated Staph proteins to bind examined. Table 6 shows the results of the optical density readings at 1/10,240 dilution of the test serum. This data suggests that Staph protein G (but not A) binds to the immunoglobulin of all of the species tested. There were considerable differences in the amount of binding among the species; this may either reflect differences in the binding of the Staph protein G or in the levels of IgG in the serum among these animals.

Table 6. Optical density values reflective of the binding of Staph Ptn A or G to IgG of cattle and game-farmed species

Species	Optical Density in ELISA	
	Staph Ptn A	Staph Ptn G
Bovine	0.086	1.584
Elk	0.014	1.835
Alpaca	0.463	1.255
White-tail Deer	0.005	1.903
Muledeer	0.000	0.746
Caribou	0.017	1.961
Llama	0.981	1.434
Bison	0.203	0.446

5. Assessment of the Midlands BioProducts Corp. Quick Test for IgG Measurement in Calves

Midlands BioProducts Corp., a US company, has recently released to the marketplace a kit designed for assessment of IgG levels in calves. The kit is immunodiffusion based, rapid and inexpensive. The kit is designed to test for failure of passive transfer of immunoglobulin from the colostrum to the serum of the neonatal calf. The test is designed to indicate whether the calf has achieved 10 g/L of serum IgG, the levels normally considered consistent with health or levels less than 10 g/L.

Midlands' kits for assessment of cattle IgG were purchased and used to test a series of serums from calves. The serums were selected to be just above or below the "cut-off" level of 10 g/L. Table 1 shows the result of this testing. The results of testing strictly according to the manufacturer's directions and testing using modifications in our laboratory are shown in Table 7. The initial testing showed good but not excellent correlation with our RID and laboratory-based ELISA measurements of the IgG levels. The samples were re-tested using calibrated pipettes and the results showed improved correlation.

We have developed a collaboration with the scientists at Midlands BioProducts and they have supplied us with the purified IgG standards used in the development of their rapid assays. We have tested those reagents in our conventional and universal bovine IgG ELISA formats. The results obtained have been very close to those predicted by the manufacturer, thus the results of the quick test can be used as estimates of the more quantitative tests developed in our laboratory.

Table 7 Results of testing calf serums with Midlands BioProducts Corp. Quick Test for IgG

Samples	RID result	Midland Kit *	Midland Kit **	Correlation
54	10.9	>10	>10	yes/yes
73	10	<10	<10	no
77	9.2	>10	<10	no/yes
186	8.6	<10	<10	yes/yes
189	12.2	>10	>10	yes/yes
189-2	8.9	>10	<10	no/yes
462	10.2	>10	>10	yes/yes

*tested according to the manufacturers directions
**tested using calibrated pipettes

6. Assessment of the Midlands BioProducts Corp. Quick Test for IgG in Game Farmed species

The Midlands kit was also applied to the testing of game-farmed species. Table 8 shows the results of testing of deer, elk , bison and alpaca serums. The results suggest that the kit as it is currently formulated may be able to detect some or all of the IgG in the serum of alpacas, bison and white tail deer. The age of the animals from which these samples were taken is unknown so the negative results in some species are not proof of failure to detect immunoglobulin.

Table 8. Results of testing serum of game-farmed species with Midlands BioProducts Quick Test for Calf IgG

Samples	Midland Kit value
A-12 Alpaca	>10
B-1 Bison	>10
C-1 Caribou	<10
E-P25 Elk	<10
M-12 Mule deer	<10
W-67 WT deer	>10

*tested according to the manufacturers directions
**tested using calibrated pipettes

7. Development of a Prototype Test for Rapid IgG Measurement

We have developed a prototype method for a rapid test for the determination of serum IgG levels. In the prototype, Staph protein G is coated to colored colloidal gold molecules, which are fixed on a strip of chromatography paper. A drop of test serum is applied to the paper. The IgG in the serum forms an immunoglobulin-Staph Protein G-gold particle complex. This complex migrates along the paper and is until trapped by an additional anti-antibody reagent (or Staph Protein G) that had been coated to the paper at a given location. The tapping of the complex allows the visualization of the particles through concentration of the colored gold beads (see diagram –appendix F).

The chromatographic paper is coated in 3 zones. Protein G is fixed to colloidal gold molecules and fixed to one of the zones (capture zone). A second area (test zone) of the paper is coated with antibody specific for the immunoglobulin of interest or of unconjugated Staph protein G. The third zone on the paper is coated with purified species immunoglobulin and serves as control line for the migration of the protein G-gold particle complexes. A drop of serum or test substance is dropped in the first zone area and in positive samples immunoglobulin-protein G-gold particle complex is formed. This complex migrates along the paper and is eventually trapped in the capture/test zone. The tapping of the complex allows the visualization of the gold particles in this area. In a negative sample the protein G-gold complexes will be trapped in control zone by the coated antibody. The test prototype would require optimization with respect to the amounts of each reagent used to enable semi-quantitative results. This test system has been tested in preliminary experiments with bovine serum. The results showed the IgG in serum could be detected by this method, however the technique was not sufficiently refined and standardized to be semi-quantitative (discriminate between serums containing above and below 10g/L).

8. Development of Conventional Enzyme Linked Immunosorbent Assays for Elk, Bison and Deer IgG.

The assay conditions for development of an enzyme-inked immunosorbent assay (ELISA) for IgG in the serum of elk, bison and deer have been established and validated.

The species standard serums and purified IgG reagents and antisera from Dr Ogunremi of the CFIA were used in standardization and validation of the ELISAs. The antisera to each species IgG was coated onto the ELISA plate to act as the immunoglobulin capture reagent and a second reagent (antisera to immunoglobulin conjugated to an enzyme) was used as the detection system for each of the target species.

The optimal concentrations for each reagent were determined by testing each in a series of doubling dilutions in a "checkerboard' titration against the other reagent. The net values are optical density of the test sample minus the value obtained in absence of the serum. The standard procedure for each of the ELISAs is detailed in the appendices.

As for cattle, the development of these ELISAs provides significant advantages over the RID method in terms of speed and reduced reagent costs and technical time.

10. Development of a "Universal" ELISA for IgG of Game Farmed Livestock

The conventional "antigen capture" ELISA as outlined above for IgG measurement in the game farmed species relies upon two antisera specific to the IgG of the species being tested. While there are a variety of reagents commercially available for the IgG of cattle, there are few or no reagents for other species, particularly for the valuable game-farmed species. In order to develop an ELISA which is widely applicable to other species it is desirable is to substitute other IgG-binding proteins for the species specific antisera in use in the conventional antigen capture ELISA.

As described for development of this test in cattle, we have systemically replaced the species-specific antisera to bovine IgG with Staph proteins. Staph Protein G (but not protein A) was found to be more potent as both capture and detection reagents than the species-specific antisera. Cross titrations established that very high dilutions of reagents were effective in detection and quantification of IgG of all of the target species

The method details of the methods developed for each of the target species is described in the appendices.

Discussion of Results

1. Identification of Optimal Reagents for Radial Immunodiffusion (RID) based IgG measurement in Cattle:

The most important consideration in the selection of the standards is the accuracy with which the levels of the immunoglobulin in the standards have been determined. There is no means by which to readily determine if the manufacturers' claims for the levels of IgG in serum samples or purified IgG preparations are accurate. For the purposes of this study, we assumed that the manufacturers' claims were likely accurate if the results obtained in RID tests using the reagents of the majority of other manufacturers was within 10-15 % of the claim.

The results of our tests showed moderate to good agreement, both among the various RID tests and with the manufacturers claims for many of the standards; while a few standards measured either significantly higher or lower than claimed by the manufacturer in most heterlogous assays.

The results of the heterologous assays most closely correspond to manufactures claims for the standard serums from Bethyl, VMRD, and the whole IgG purified preparations from ICN and Jackson. The standards from Biogenesis and Chemicon did not measure within 10-15 % of their claims when using most other manufacturers reagents and controls.

Other important considerations in the selection of the standard are the subclass(es) of immunoglobulin represented in the standard. Bovine serum contains 3 subclasses of immunoglobulin G: IgG1, IgG2 and IgG3. The relative proportions of the subclasses of IgG1:IgG2 in serum of the adult cow have been reported to be similar while milk and colostrum are reported to have higher levels of IgG1. Little is known about the proportions of IgG3, however these levels are low compared to IgG1 and IgG2. It is important in selection of standards that the standards contain representative proportions of the IgG subclasses. Among the standards tested in this study some are purported to contain both IgG1 and IgG2 while others are subclass specific. It was considered critical to test to ensure that the regents used in our assay would detect the major proportions of both of the major IgG subclasses of cattle. According to the results of our studies, the best standards to use for estimation of total bovine IgG (those with both IgG 1 &2) are the standards from Bethyl and Jackson Laboratories.

The antisera used in the RID (and ELISA) methods are also important since they must be capable of detection of all subclasses and alloytpes of IgG. Allotypes are slight variations in the IgG molecule from individual to individual. The IgG1 and IgG2 subclasses are known to express allotypic determinants, the best-described variants are the IgG2 A1 and A2 allotypic determinants (now designated $IgG2^a$ and $IgG2^b$, respectively. The presence of IgG subclasses and allotypes within subclasses is an important consideration for analysis of bovine immunoglobulins since heterologous antisera raised to IgG will be biased toward detection of the immunizing isotypes/allotypes. For example, antisera raised in rabbits to IgG2 of the A2 allotype ($IgG2^b$) may contain activity mainly directed to A2 and may fail to detect IgG2 of the A1 allotype ($IgG2^a$). Less is known about the impact of IgG1 allotypes, the structural

differences between serum and colostral IgG1, and the detection (or failure of detection) of IgG3, however it is likely that the antigenic differences will be great enough to bias quantification.

The issue of allotype bias was considered to be potentially important in the measurement of bovine IgG using heterologous polyclonal antisera to the H chain of IgG (as in RIDs or ELISA) since the reagent bias may result in underestimation of the IgG levels. Thus it is possible that H chain specific reagents whether class or subclass specific may fail to detect a proportion of the total IgG (due to failure to detect IgG3 or the nonimmunizing allotypes of IgG1 and IgG2). The commercially available kits for detection of bovine IgG by RID have H chain only specific antisera thus it was suspected that these may underestimate the levels found in some samples. In the present study, one of the H chain specific reagents performed poorly however 2 other kits gave results similar to alternative reagents. The use of a combination of two kits, the IgG1 and IgG2 specific kits from Bethyl Laboratories gave the best results, finding almost all standards testing within 10% of the manufactures claims, however it is expensive and labor intensive to utilize 2 separate assays for each sample tested.

Antisera to bovine IgG, in addition to the epitopes found on the class-specific heavy chains also have epitopes on the light chains, which, in cattle, are predominately (90%) lambda. Given the bias in heterolgous antisera raised to IgG H chain isotypes an alternative strategy which may more accurately measure IgG levels in all individuals, is to use reagents directed to the light as well as heavy chains. In the present study there was no obvious difference in results when H&L reagents were compared to H chain reagents used in the kits, however only a few samples were examined in this study which may not have been adequate to demonstrate allotypic bias.

In summary, it appears the IgG standard serums or purified IgG preparations from either Bethyl or Jackson Laboratories could be used as the species standard to develop assays for bovine IgG measurement. The IgG (H &L) antisera from several commercial sources including Jackson, Bethyl, ICN or Sigma appear to have similar efficacy in RID assays.

This is the first study, which has examined the commercially available reagents for determination of bovine IgG levels. The data is useful since there has been difficulty in comparing among various studies the results of IgG measurements because the methods used for this determination have been variable and often inaccurate. The results of this study have important implications for standardization of bovine IgG testing. As a consequence of this study the reagents in current use in the Diagnostic Immunology laboratory of the Prairie Diagnostic Laboratory at the WCVM have been changed. A summary of these finding were presented to the National Veterinary Services Laboratory of the US Department of Agriculture in Ames Iowa and were presented to the participants of the USDA Antibody Products Public Meeting in Ames Iowa in April. The data will be used to encourage other researchers and manufacturers to develop standard assays for measurements of bovine IgG to enable more meaningful comparisons among studies from different laboratories.

2. Development of an Enzyme Linked Immunosorbent Assay (ELISA) for Detection of IgG in Cattle and Game-farmed Livestock Species

The ELISA for IgG detection is an "antigen-capture" assay, which requires 2 antisera directed against the IgG; this is in contrast to the RID method that requires only one antisera. In the ELISA, one antisera acts to "capture" the bovine IgG from the sample and a second antisera (labeled with the enzyme) detects the captured immunoglobulin. The technical requirements for the antisera are similar to those for the RID, the antisera must bind to all subclasses of the bovine IgG and not to other proteins in the serums. In addition, to be useful in ELISA, the antisera must not bind to the immunoglobulins in the other antisera used in the assay (so that the capture and detection antiseras do not bind to each other. The latter was shown to be the major limitation in selecting reagents for the IgG antigen capture ELISA. Several reagents tested were eliminated based on binding either to the other antisera or to proteins in fetal calf serum.

We have developed an ELISA for bovine, elk, deer and bison IgG, which can be used to determine the immunoglobulin, levels in serum samples.
The assay can be used to replace the RID method currently in use in cattle and provide the first method available for performing these measurements in the other target species.

The ELISA offers advantages over the previous method used in cattle. The test can be performed in about 2 hours while the RID method requires over 24 hours. In the RID method a maximum of 20 samples can be tested /plate while with the ELISA over 40 samples can be tested/plate. Thus while the RID, because it is the conventional test, still may be the gold standard test for official/legal purposes, the ELISA is faster and less expensive in reagents and technical time. Initially the ELISA will be most useful for diagnostic and research projects, but with time will probably replace the conventional RID methods.

3. Development of a "Universal" ELISA for Detection of IgG in the Serum of Cattle and Game Farmed Species

We found that the Staph protein G binds to bovine immunoglobulin with higher affinity than the conventional antisera raised to bovine IgG and thus can be substituted for antisera in ELISA assays for bovine IgG. The technical details of the assay using Staph protein G are described in Appendix C.

The Staph protein G has some advantages over the conventional antisera for use in cattle for measurement of IgG. Staph protein G can be used at higher dilutions therefore is less expensive; it will not demonstrate allotype biases to the immunizing allotypes (as might be expected with antisera) and unlike antisera (which are available in finite amounts) can be purchased in unlimited quantities. We have demonstrated that Staph protein G binds to the IgG of all of the target game-farmed species and thus an assay based on the use of this reagent has been standardized for use in each species. This assay is of benefit since there are no commercially available sources of these reagents and those provided for research purposes for the present study are of finite quantity. In addition the present studies. in demonstrating the "universality" of the

Staph G reagent, suggests that the production of a kit for use in the neonates of all species is technically possible.

7. Development of a Rapid Method for Detection of IgG

We have experimented with the development of a prototype assay for the measurement of IgG in calf serum. The prototype is based on a reaction on chromatography paper on which the 'capture reagent' is Staph Protein G coupled to colored gold beads and the detection reagent is either antiserum to bovine IgG or Staph protein. The capture and detection reagents are fixed to the "zones" on the paper. This is easily achieved simply by dotting the reagent on the paper and allowing it to dry.

The serum containing the IgG is applied by drops which wick up the paper. The Staph protein G –gold beads bind the IgG and the entire complex moves along the paper until the IgG is "captured" –the reaction is visible because of concentration of the gold beads. Similar assays are in use for other purposes (for examples for detection of heartworm antigen in the serum of dogs).

While demonstrating proof of principle is straightforward, developing a commerical kit that is accurate, robust and reliable is complex and expensive. Such kits must be licensed by the CFIA and USDA. Licensing of diagnostic kits is an expensive and lengthy procedure. The introduction to the marketplace of the Midland BioProducts Corp. kit makes further pursuit of development this assay less attractive and unlikely to attract commercial investment. The Midland kit also has patent pending that might preclude the use of a similar kit if successful. It has been our strategy to develop a mutually beneficial collaborative relationship with Midlands BioProducts.rather than to continue independent pursuit of a competing kit.

Personnel:

Some of the technical work for this project has been accomplished by Ms Carrie Gress, a final year student completing an honors degree in Microbiology. As Ms Gress received course credit for her work on this project there was no salary payable to her for this work. Ms Gress worked about 20 hours/week in this study until April/99. Ms Gress was assisted in her laboratory training by Mr Ron Sargent and Mr Brain Chelack, however as Mr Chelack is a co-applicant in the study (and an employee of the Prairie Diagnostic Service) only salary costs for Mr Sargent were partially covered by this grant. Ms Marnie Anderson was the technical personnel whom completed the project, working from Sept/00-Jan/01 approximately 1/3 time. Her salary costs relating to this study were covered from a discretionary income account.

Equipment: There has been no purchase of equipment for this study.

Project Developed Material: none available.

Project Photos: none available

Acknowledgements: the ADF was be acknowledged when the results of the study were presented to the university community in April/2000 as part of Ms Gress' course work.

Expense statement: this has been directly forwarded to your offices from the U of S Research Services. Note that there has been a deviation from the original proposed budget in that a higher proportion of funding has been used for reagents and less for salary. This considered necessary when it was discovered early in the study that there were discrepancies among the findings with different commercial standards for bovine IgG in the assay. Since it was considered critical to establish the tests with valid standards considerable time and expense was incurred to ensure that the most accurate standards were chosen.

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Development of a Universal Assay for Measuring Immunoglobulin Levels in Cattle and Game Framed Livestock

Deborah Haines

Appendices

Radial Immunodiffusion (RID) Assay for the Detection of IgG.

A. Material and Supplies

1. Immunodiffusion Plates (8.4 cm x 9.4 cm) (Pharmacia).
2. Seakem ME Agarose (FMC Bioproducts 50012).
3. Phosphate Buffered Saline (Gibco BRL, 21600-010).
4. POUR Sterile Water (Abbott Lab. E319)
5. Falcon centrifuge tube 50 mL (Becton Dickinson, 2098)
6. Sodium azide, NaN3, (Fisher S-227).
7. pH Meter and glass Combination Electrode with Calomel Reference.
8. Pipetters capable of delivering 10-1000 µL (Pipetteman p10, p200, p1000).
9. Microwave Oven (GE SpaceMaker II)
10. Waterbath at 56 deg C
11. Agar Punch Kit, with punch (2.0 mm) and templates (Pharmacia Biotech 80-1109-69,70,71).
12. RID Plate Reader (Transdyne General Corp. 2743).
13. Glass Culture Tubes (16 x 100 mm) (Fisher 60825-423)
14. Anti-immunoglobulin G to be mixed with the gel matrix: (Rabbit anti bovine IgG (H and L chain) (Jackson Labortories 301-001-003).
15. Reference Reagents:
    Bovine IgG Standard (Chrom-Pure Bovine IgG 001-000-003, Jackson Laboratories).
    Reference colostrum (Serial Number DHT001 reconstituted according to package directions, aliquoted at 100 µL/aliquot and stored at -20 deg C)
    Reference Serum of known IgG concentration (Bethyl Laboratories, Bovine Ref. Serum RS10-103-3)
16. Test Serial (colostrum) and/or bovine serums
17. Levelling Set (Pharmacia 18-1016-88)

B. Equipment

1. pH Meter and Glass Combination Electrode with Calomel Reference
2. Agar Punch Kit, with punches (Pharmacia)
3. RID Plate-reader (Transdyne General Corp. 2743)
4. Levelling Set (Pharmacia 18-1016-88)
5. Computer and software program for linear regression analysis (IBM 486 with Lotus 123)

6. Vortex mixer (American Scientific Products).
7. Laboratory Balance (Sartorius)
8. Vaccum pump (Doerr Electric Corp).

C. Hazards

1. Handling of Sodium Azide (NaN3):
Avoid breathing of sodium azide dust. Avoid contact with skin, eyes, and clothing. Reacts with acids by emitting toxic fumes.

D. QA and Critical Control Points (CCP):

1. PBS Buffer Solution pH = 7.25 $\pm$ 0.1 (see section G for preparation)
2. Accurate dilution of the anti-immunoglobulin G.
3. Accurate preparation and dispensing of the agarose.
4. Volume of agarose/plate = 15.0 $\pm$ 0.5 mL
5. Careful cutting of the wells and removal of the agarose.
6. Accurate reconstition and dilution of test samples.
7. Level of filled wells: accurate dispensing of 4 µL.
8. Incubation temperature = 20-25 deg C and humidity levels $\geq$ 40%.
9. Careful reading of ring diameters.
10. Careful calculation of the regression line and results.

E. Method:

1. Prepare 1.0% Seakem ME Agarose by dissolving 0.15 g agarose in 15.0 mL PBS (pH = 7.25) in a 50 mL Falcon tube. Place the screw cap on loosely and bring to a boil in the microwave oven (full power for 55 seconds).
2. Cool the agarose solution to 56 deg C using a water bath.
3. Dispense 14.55 $\pm$ 0.1 mL of agarose solution into 16 x 100 mm culture tubes. Add 10 µL of 10% sodium azide (10 gms of NaN3 in 100 mLs of POUR water).
4. Add 0.45 mL of the rabbit anti-immunoglobulin G (Jackson Laboratories) into each tube and mix well to ensure a homogeneous mixture without bubbles.
5. Select an empty immunodiffusion plate and place it on a levelled table. Immediately pour the melted agar mixture (15 mL) into the plate. Allow the agar to solidify at room temperature (20-25 deg C) for 15 minutes.
6. Place the plate in a humidified chamber (9 x16 inch tupperware container carpeted with moistened paper towels). Incubate for 30 minutes.
7. Using a gel punch (2.0 mm), RID template and vaccum pump, cut and aspirate wells in the agar plate. The vaccum pump should be set to draw 8-10 inches Hg. Each well should be 2.0 mm diameter (about 6 µL volume) and separated from its neighbours by 13 mm.
8. Store the plate in a humid chamber until application of reagents and samples. Plates should be used on the day prepared.
9. Prepare serial 2 fold dilutions (1:4-1:32) of the Bovine IgG Standard (Bovine Immunoglobulin G, Jackson Laboratories) to obtain immunoglobulin concentrations of approximately 0.86, 1.73, 3.45, and 6.9 g/L using PBS (pH 7.25).

10. Prepare dilutions of the following controls in PBS (pH 7.25):
    Reference Bovine Serum (Bethyl Laboratories) (diluted 1/8)
    Reference Colostrum (Serial # DHT 001) (diluted 1:15)
11. Prepare samples of the test serial (350 g of the product plus 750 mL POUR water to make a total of approximately 1 L) or serums (diluted 1/2 in PBS (pH 7.25).
11. Prepare a 1/15 dilution of the reconstituted test serial colostrum using PBS (pH 7.25).
12. Dispense 5 replicates of 4 µL of the diluted reference colostrum (DHT 001) and reference serum (Bethyl Laboratories) on the plate.
13. Dispense 2 replicates of 4 µL of each dilution of the Bovine IgG Standard (Jackson Laboratories) for the standard curve.
14. Dispense 2 replicates of 4 µL of the diluted test serial and/or test serums on the plate.
15. Incubate all plates at room temperature (20-25 deg C) for 18 hours in a humid chamber.
16. Using the plate reader, measure and record the ring diameters for the precipitin rings surrounding the wells.
17. Using the results (ring diameters) obtained for each of the 2 fold dilutions (1:4-1:32) of the Bovine IgG Standard (Bovine Immunoglobulin G, Jackson Laboratories) generate a regression line (for each plate) for the variables R (ring diameter) vrs log10 (conc).
18. The plate is acceptable if the R2 is $\geq$ 0.97 for the standard curve and the values obtained for the reference colostrum and reference serum are the expected values $\pm$ 10%.
19. Ig concentration for the test serial and/or serums is determined using the regression line of the Bovine IgG Standard obtained for each plate and the average of the observed (measured) ring diameters for the sample.
20. The values obtained for the replicate tests of the reference colostrum and serum are acceptable if at least 4/5 values are less than $\pm$ 10% the mean of the five values. The value obtained for the reference serum must be +10% of the manufacturers claim.

Bovine IgG ELISA

Intoduction:

The highest numbers of death losses in cattlie and other farmed veterinary species is from infectious disease during the first 30 days of life. Many studies have documented that this increased risk of infectious disease during the neonatal period is directed associated with inadequate passive transfer of maternal colostral immunologlobulin. The measurement of serum immunoglobulin levels can be used clinically to identify neonates with inadequate passive transfer of immunoglobulin. Knowledge of the neonates' immunological status can then be used in making decisions regarding the prognosis and the treatment of individual animals and in providing suggestions for herd husbandry and management practices.

Objective:

To develop an enzyme linked immunoassay (ELISA) for measurement of Bovine IgG

A. Equipment

1. BioRad Microplate Reader, (BioRad)
2. Pipettes-capeable of delivering 1- 10000ul, (Eppendorf, Pipettman)
3. Pipette Tips 1- 10000 ul volume, (BioRad, VWR)
4. Refrigerator (Kelvinator)

B. Material and Supplies

1. ELISA Dynex Immulon IV plates, (VWR)
2. Polypropylene Culture Tubes, Polypropylene 12x75 mm and 17x100mm, (VWR)
3. TMB, (Gibco-Life Technologies)
4. Tween 20, (VWR)
5. Trisma base (Sigma)
8. Sheep x-Bovine IgG-affinity purified (Bethyl, Lot # A10-118A-4)
9. Bovine Reference Serum, IgG concentration=21.3 mg/ml (Bethyl, Lot # RS10-103-3)
10. Sheep x-Bovine IgG HRP (Bethyl, Lot # A10-118P-5)
11. Sodium Chloride (BDH Inc.)
12. Gelatin, (Difco)

C. Solution Preparation:

1. 5M Sodium Chloride, (BDH)
   -Add 292.2g into 1000ml of Distilled Water.
2. 1M Tris, (Sigma)
   -Add 60.55g into 500ml of Distilled Water.
   -pH solution with 1M Hydrochloric Acid, pH 8.0, (AnalaR).

3. Coating Buffer- 0.05M Sodium carbonate, pH 9.6
    -Add 0.2g Sodium Azide, NaN3, (BDH); 2.94g Sodium Bicarbonate, NaHCO3, (BDH); and 1.60g Sodium Carbonate, Na2CO3, (BDH) to 500 ml of distilled water.
    -Dissolve ingredients by mixing.
    -Adjust to a pH of 9.6 with Sodium Hydroxide.
    -Adjust solution to a final volume of 1000ml with distilled water.
4. Wash Solution- 50mM Tris, O.14 M NaCl, 0.5% Tween 20, pH 8.0
    -Add 28 ml of 5M NaCl; 50 ml 1M Tris; and 5 ml Tween 20 to a 1000 ml Erylenmeyer flask.
    -Adjust pH to 8.0.
    -Adjust solution to a final volume of 1000 ml with Distilled Water.
5. Postcoat Solution- 50mM Tris, O.15 M NaCl, 0.2% Gelatin, pH 8.0
    -Add 1.5 ml of 5M NaCl; 2.5 ml of 1M Tris; and 0.1 g of Gelatin into a 50 ml Erylenmeyer flask.
    -Adjust pH to 8.0.
    -Adjust solution to a final volume of 50 ml with Distilled water.
6. Sample/Conjugate Diluent- 50mM Tris, O.15 M NaCl, 0.2% Gelatin, 0.5% Tween 20, pH 8.0
    -Combine 3.0 ml of5M NaCl with 5 ml 1M Tris, 0.2 g Gelatin, and 500 ul of Tween 20 in a 100 ml Erylenmeyer flask.
    -Adjust pH to 8.0.
    -Adjust final volume with Distilled Water (100 ml)
7. Stop Solution- 1M Sulpuric Acid, H2SO4, (AnalaR)
    -Add 29.1 ml into 970.9 ml Distilled Water

D.Method:

1.Dilute sheep x-Bovine IgG-affinity purified antibody 1:100 with Coating Buffer. Transfer 100ul into each well.
2.Incubate at 4 C overnight.
3.After incubation, dump Coating Solution from the plate.
4.Wash each well with 200 ul of Wash Solution. Repeat three more times.
5.Add 200ul of Postcoat Solution to each well.
6.Incubate 30 minutes at room temperature.
7.Repeat step 4.
8.Dilute serum samples in Sample/Conjugate Diluent. Make an initial dilution of 10 ul of serum into 990ul of Sample/Conjugate Diluent. Take 10 ul of previous dilution and add it in 990 ul of diluent. Take 100 ul of previous dilution and add it to 900 ul of diluent. Final dilution of the serum sample is a 1:200 000. For Colostrum samples make a further 2 fold dilution in diluent for a dilution factor of 1:400 000.
9.Transfer 100ul of the diluted serum sample to predetermined wells.
10.Incubate 60 minutes at room temperature.
11.After incubation, wash each well as in step 4, but repeat two more times.
12.Dilute the sheep x-Bovine IgG-HRP Conjugate by pipetting 1 ul of conjugate into 5ml of diluent, then transfer 1 ml into 9 ml of diluent for a final dilution of 1:50 000.

13.Transfer 100ul of the Antibody/HRP Conjugate into each well.
14.Incubate the plate for 60 minutes at room temperature.
15.Repeat step 11.
16.Prepare the Substrate Solution by mixing equal volumes of the two TMB subtrate reagents.
17.Transfer 100ul of Substrate Solution to each well.
18.Incubate plate for 30 minutes at room temperature.
19.Add 50ul of Stop Solution to each well.
20.Using a microtiter plate reader, read the O.D.'s of the wells at 450nm.

Bison IgG ELISA

Objective:
To develop an enzyme linked immunoassay (ELISA) for measurement of Bison IgG

Material, Supplies and Methodology

A. Equipment

1. BioRad Microplate Reader, (BioRad)
2. Pipettes-capeable of delivering 1- 10000ul, (Eppendorf, Pipettman)
3. Pipette Tips 1- 10000 ul volume, (BioRad, VWR)
4. Refrigerator (Kelvinator)

B. Material and Supplies

1. ELISA Dynex Immulon IV plates, (VWR)
2. Polypropylene Culture Tubes, Polypropylene 12x75 mm and 17x100mm, (VWR)
3. TMB, (Gibco-Life Technologies)
4. Tween 20, (VWR)
5. Trisma base (Sigma)
8. Bison IgG (1.92 ug/ul, Dele Ogunremi)
9. Rabbit anti-Bison IgG-AP and rabbit anti-Bison IgG HRP (Dele Ogunremi)
10. Rabbit anti-Bison (Dele Ogunremi)
11. Sodium Chloride (BDH Inc.)
12. Gelatin, (Difco)

C. Solution Preparation:

1. 5M Sodium Chloride, (BDH)
   -Add 292.2g into 1000ml of Distilled Water.
2. 1M Tris, (Sigma)
   -Add 60.55g into 500ml of Distilled Water.
   -pH solution with 1M Hydrochloric Acid, pH 8.0, (AnalaR).
3. Coating Buffer- 0.05M Sodium carbonate, pH 9.6
   -Add 0.2g Sodium Azide, NaN3, (BDH); 2.94g Sodium Bicarbonate, NaHCO3, (BDH); and 1.60g Sodium Carbonate, Na2CO3, (BDH) to 500 ml of distilled water.
   -Dissolve ingredients by mixing.
   -Adjust to a pH of 9.6 with Sodium Hydroxide.
   -Adjust solution to a final volume of 1000ml with distilled water.
4. Wash Solution- 50mM Tris, O.14 M NaCl, 0.5% Tween 20, pH 8.0
   -Add 28 ml of 5M NaCl; 50 ml 1M Tris; and 5 ml Tween 20 to a 1000 ml Erylenmeyer flask.
   -Adjust pH to 8.0.
   -Adjust solution to a final volume of 1000 ml with Distilled Water.
5. Postcoat Solution- 50mM Tris, O.15 M NaCl, 0.2% Gelatin, pH 8.0

-Add 1.5 ml of 5M NaCl; 2.5 ml of 1M Tris; and 0.1 g of Gelatin into a 50 ml Erylenmeyer flask.
-Adjust pH to 8.0.
-Adjust solution to a final volume of 50 ml with Distilled water.

6. Sample/Conjugate Diluent- 50mM Tris, O.15 M NaCl, 0.2% Gelatin, 0.5% Tween 20, pH 8.0
-Combine 3.0 ml of5M NaCl with 5 ml 1M Tris, 0.2 g Gelatin, and 500 ul of Tween 20 in a 100 ml Erylenmeyer flask.
-Adjust pH to 8.0.
-Adjust final volume with Distilled Water (100 ml)

7. Stop Solution- 1M Sulpuric Acid, H_2SO_4, (AnalaR)
-Add 29.1 ml into 970.9 ml Distilled Water

D. Method:

1.Dilute rabbit-anti-bison antibody 1:1600 with Coating Buffer. Transfer 100ul into each well.
2.Incubate at 4C overnight.
3.After incubation, dump Coating Solution from the plate.
4.Wash each well with 200 ul of Wash Solution. Repeat three more times.
5.Add 200ul of Postcoat Solution to each well.
6.Incubate 30 minutes at room temperature.
7.Repeat step 4.
8.Dilute the standards and samples in Sample/Conjugate Diluent:
Standard- Make an initial dilution of 1ul into 5900 ul (100 ng/ml). From here make 2-fold dilutions (1ml + 1ml) for standards 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78 ng/ml.
Sample- Make an initial dilution of 10 ul of sample serum into 990ul of Sample/Conjugate Diluent. Take 10 ul of previous dilution and add it in 990 ul of diluent. Take 80 ul of previous dilution and add it to 1920 ul of diluent. Final dilution of the serum sample is a 1:250 000. For Colostrum samples make a further 2 fold dilution in diluent for a dilution factor of 1:500 000.
9.Transfer 100ul of standard and sample to predetermined wells.
10.Incubate 60 minutes at room temperature.
11.After incubation, wash each well as in step 4, but repeat two more times.
12.Dilute the rabbit-anti-Bison IgG HRP Conjugate by pipetting 5 ul of conjugate into 5ml of diluent (1/1000; 3.9 ug/ml).
13.Transfer 100ul of the Antibody/HRP Conjugate into each well.
14.Incubate plate for 60 minutes at room temperature.
15.Repeat step 11.
16.Prepare the Substrate Solution by mixing equal volumes of the two TMB subtrate reagents.
17.Transfer 100ul of Substrate Solution to each well.
18.Incubate plate for 30 minutes at room temperature.
19.Add 50ul of Stop Solution to each well.
20.Using a microtiter plate reader, read the O.D.'s of the wells at 450nm.

ELK IgG ELISA

Objective:

To develop an enzyme linked immunoassay (ELISA) for measurement of Elk IgG

Materials and Supplies:

A. Equipment

1. BioRad Microplate Reader, (BioRad)
2. Pipettes-capeable of delivering 1- 10000ul, (Eppendorf, Pipettman)
3. Pipette Tips 1- 10000 ul volume, (BioRad, VWR)
4. Refrigerator (Kelvinator)

B. Material and Supplies

1. ELISA Dynex Immulon IV plates, (VWR)
2. Polypropylene Culture Tubes, Polypropylene 12x75 mm and 17x100mm, (VWR)
3. TMB, (Gibco-Life Technologies)
4. Tween 20, (VWR)
5. Trisma base (Sigma)
8. Elk IgG (1.92 ug/ul, Dele Ogunremi)
9. Rabbit anti-Elk IgG-AP and rabbit anti-Elk IgG HRP (Dele Ogunremi)
10. Rabbit anti-Elk (Dele Ogunremi)
11. Sodium Chloride (BDH Inc.)
12. Gelatin, (Difco)

C. Solution Preparation:

1. 5M Sodium Chloride, (BDH)
   -Add 292.2g into 1000ml of Distilled Water.
2. 1M Tris, (Sigma)
   -Add 60.55g into 500ml of Distilled Water.
   -pH solution with 1M Hydrochloric Acid, pH 8.0, (AnalaR).
3. Coating Buffer- 0.05M Sodium carbonate, pH 9.6
   -Add 0.2g Sodium Azide, NaN3, (BDH); 2.94g Sodium Bicarbonate, NaHCO3, (BDH); and 1.60g Sodium Carbonate, Na2CO3, (BDH) to 500 ml of distilled water.
   -Dissolve ingredients by mixing.
   -Adjust to a pH of 9.6 with Sodium Hydroxide.
   -Adjust solution to a final volume of 1000ml with distilled water.
4. Wash Solution- 50mM Tris, O.14 M NaCl, 0.5% Tween 20, pH 8.0
   -Add 28 ml of 5M NaCl; 50 ml 1M Tris; and 5 ml Tween 20 to a 1000 ml Erylenmeyer flask.
   -Adjust pH to 8.0.
   -Adjust solution to a final volume of 1000 ml with Distilled Water.
5. Postcoat Solution- 50mM Tris, O.15 M NaCl, 0.2% Gelatin, pH 8.0
   -Add 1.5 ml of 5M NaCl; 2.5 ml of 1M Tris; and 0.1 g of Gelatin into a 50 ml Erylenmeyer flask.
   -Adjust pH to 8.0.

-Adjust solution to a final volume of 50 ml with Distilled water.

6. Sample/Conjugate Diluent- 50mM Tris, O.15 M NaCl, 0.2% Gelatin, 0.5% Tween 20, pH 8.0
   -Combine 3.0 ml of5M NaCl with 5 ml 1M Tris, 0.2 g Gelatin, and 500 ul of Tween 20 in a 100 ml Erylenmeyer flask.
   -Adjust pH to 8.0.
   -Adjust final volume with Distilled Water (100 ml)
7. Stop Solution- 1M Sulpuric Acid, H2SO4, (AnalaR)
   -Add 29.1 ml into 970.9 ml Distilled Water

Method:

1.Dilute rabbit-anti-Elk antibody 1:1600 with Coating Buffer. Transfer 100ul into each well.
2.Incubate at 4 C overnight.
3.After incubation, dump Coating Solution from the plate.
4.Wash each well with 200 ul of Wash Solution. Repeat three more times.
5.Add 200ul of Postcoat Solution to each well.
6.Incubate 30 minutes at room temperature.
7.Repeat step 4.
8.Dilute serum samples in Sample/Conjugate Diluent. Make an initial dilution of 10 ul of serum into 990ul of Sample/Conjugate Diluent. Take 10 ul of previous dilution and add it in 990 ul of diluent. Take 100 ul of previous dilution and add it to 900 ul of diluent. Final dilution of the serum sample is a 1:200 000. For Colostrum samples make a further 2 fold dilution in diluent for a dilution factor of 1:400 000.
9.Transfer 100ul of the diluted serum sample to predetermined wells.
10.Incubate 60 minutes at room temperature.
11.After incubation, wash each well as in step 4, but repeat two more times.
12.Dilute the rabbit-anti-Elk IgG HRP Conjugate by pipetting 10 ul of conjugate into 10ml of diluent (1/1000).
13.Transfer 100ul of the Antibody/HRP Conjugate into each well.
14.Incubate the plate for 60 minutes at room temperature.
15.Repeat step 11.
16.Prepare the Substrate Solution by mixing equal volumes of the two TMB subtrate reagents.
17.Transfer 100ul of Substrate Solution to each well.
18.Incubate plate for 30 minutes at room temperature.
19.Add 50ul of Stop Solution to each well.
20.Using a microtiter plate reader, read the O.D.'s of the wells at 450nm.
21.Compare O.D readings to a standard curve. The curve was constructed by testing the average O.D. readings among 16 normal Elk serum samples:

O.D @ 450	Standard Units
1.200 - 1.300 =	500 units
1.100 - 1.000 =	250 units
0.900 - 0.800 =	125 units
0.600 - 0.500 =	62 units

Mule/White-Tail Deer IgG ELISA

Objective:

To develop an enzyme linked immunoassay (ELISA) for measurement of Mule/White-Tail Deer IgG.

Materials and Supplies:

A. Equipment

1. BioRad Microplate Reader, (BioRad)
2. Pipettes-capeable of delivering 1- 10000ul, (Eppendorf, Pipettman)
3. Pipette Tips 1- 10000 ul volume, (BioRad, VWR)
4. Refrigerator (Kelvinator)

B. Material and Supplies

1. ELISA Dynex Immulon IV plates, (VWR)
2. Polypropylene Culture Tubes, Polypropylene 12x75 mm and 17x100mm, (VWR)
3. TMB, (Gibco-Life Technologies)
4. Tween 20, (VWR)
5. Trisma base (Sigma)
8. Mule Deer IgG (Dele Ogunremi)
9. Rabbit anti-Mule Deer IgG-AP and rabbit anti-Mule Deer IgG HRP (Dele Ogunremi)
10. Rabbit anti-Mule Deer (Dele Ogunremi)
11. Sodium Chloride (BDH Inc.)
12. Gelatin, (Difco)

C. Solution Preparation:

1. 5M Sodium Chloride, (BDH)
   -Add 292.2g into 1000ml of Distilled Water.
2. 1M Tris, (Sigma)
   -Add 60.55g into 500ml of Distilled Water.
   -pH solution with 1M Hydrochloric Acid, pH 8.0, (AnalaR).
3. Coating Buffer- 0.05M Sodium carbonate, pH 9.6
   -Add 0.2g Sodium Azide, NaN3, (BDH); 2.94g Sodium Bicarbonate, NaHCO3, (BDH); and 1.60g Sodium Carbonate, Na2CO3, (BDH) to 500 ml of distilled water.
   -Dissolve ingredients by mixing.
   -Adjust to a pH of 9.6 with Sodium Hydroxide.
   -Adjust solution to a final volume of 1000ml with distilled water.
4. Wash Solution- 50mM Tris, O.14 M NaCl, 0.5% Tween 20, pH 8.0
   -Add 28 ml of 5M NaCl; 50 ml 1M Tris; and 5 ml Tween 20 to a 1000 ml Erylenmeyer flask.
   -Adjust pH to 8.0.
   -Adjust solution to a final volume of 1000 ml with Distilled Water.
5. Postcoat Solution- 50mM Tris, O.15 M NaCl, 0.2% Gelatin, pH 8.0
   -Add 1.5 ml of 5M NaCl; 2.5 ml of 1M Tris; and 0.1 g of Gelatin into a 50 ml Erylenmeyer flask.

-Adjust pH to 8.0.

-Adjust solution to a final volume of 50 ml with Distilled water.

6. Sample/Conjugate Diluent- 50mM Tris, O.15 M NaCl, 0.2% Gelatin, 0.5% Tween 20, pH 8.0

-Combine 3.0 ml of5M NaCl with 5 ml 1M Tris, 0.2 g Gelatin, and 500 ul of Tween 20 in a 100 ml Erylenmeyer flask.

-Adjust pH to 8.0.

-Adjust final volume with Distilled Water (100 ml)

7. Stop Solution- 1M Sulpuric Acid, H2SO4, (AnalaR)

-Add 29.1 ml into 970.9 ml Distilled Water

Method:

1.Dilute rabbit-anti-Mule Deer antibody 1:1600 with Coating Buffer. Transfer 100ul into each well. 2.Incubate at 4 C overnight.

3.After incubation, dump Coating Solution from the plate.

4.Wash each well with 200 ul of Wash Solution. Repeat three more times.

5.Add 200ul of Postcoat Solution to each well.

6.Incubate 30 minutes at room temperature.

7.Repeat step 4.

8.Dilute serum samples in Sample/Conjugate Diluent. Make an initial dilution of 10 ul of serum into 990ul of Sample/Conjugate Diluent. Take 10 ul of previous dilution and add it in 990 ul of diluent. Take 100 ul of previous dilution and add it to 900 ul of diluent. Final dilution of the serum sample is a 1:200 000. For Colostrum samples make a further 2 fold dilution in diluent for a dilution factor of 1:400 000.

9.Transfer 100ul of the diluted serum sample to predetermined wells.

10.Incubate 60 minutes at room temperature.

11.After incubation, wash each well as in step 4, but repeat two more times.

12.Dilute the rabbit-anti-Mule Deer IgG HRP Conjugate by pipetting 10 ul of conjugate into 10ml of diluent (1/1000).

13.Transfer 100ul of the Antibody/HRP Conjugate into each well.

14.Incubate the plate for 60 minutes at room temperature.

15.Repeat step 11.

16.Prepare the Substrate Solution by mixing equal volumes of the two TMB subtrate reagents.

17.Transfer 100ul of Substrate Solution to each well.

18.Incubate plate for 30 minutes at room temperature.

19.Add 50ul of Stop Solution to each well.

20.Using a microtiter plate reader, read the O.D.'s of the wells at 450nm.

21.Compare O.D readings to a standard curve. The curve was constructed by testing the average O.D. readings among 16 normal White-Tail Deer serum samples:

O.D @ 450	Standard Units
1.800 - 2.000 =	500 units
1.500 - 1.600 =	250 units
1.200 - 1.300 =	125 units
1.100 - 0.800 =	62 units

"Universal" Enzyme Linked Immunosorbent Assay (ELISA) for the Dectection of IgG in Cattle.

Material and Supplies:

A. Equipment
1. BioRad Microplate Reader, (BioRad)
2. Pipettes-capeable of delivering 1- 10000ul, (Eppendorf, Pipettman)
3. Pipette Tips 1- 10000 ul volume, (BioRad, VWR)
4. Refrigerator (Kelvinator)

B. Material and Supplies:
1. ELISA Dynex Immulon IV plates, (VWR)
2. Polypropylene Culture Tubes, Polypropylene 12x75 mm and 17x100mm, (VWR)
3. ABTS (Gibco-Life Technologies)
4. Tween 20, (VWR)
5. Trisma base (Sigma)
8. Bovine Reference Serum (21.3 mg/ml, Bethyl Laboratories Inc., Lot # RS10-103-3)
9. Recombinant Protein G-HRP (Zymed 1139882)
10. Sheep x-Bovine IgG-affinity purified (Bethyl Laboratories Inc., Lot # A10-118A-4)
11. Sodium Chloride (BDH Inc.)
12. Gelatin, (Difco)
13. 1 % Sodium Dodecyl Sulfate

C. Solution Preparation:
1. 5M Sodium Chloride, (BDH)
   -Add 292.2g into 1000ml of Distilled Water.
2. 1M Tris, (Sigma)
   -Add 60.55g into 500ml of Distilled Water.
   -pH solution with 1M Hydrochloric Acid, pH 8.0, (AnalaR).
3. Coating Buffer- 0.05M Sodium carbonate, pH 9.6
   -Add 0.2g Sodium Azide, NaN3, (BDH); 2.94g Sodium Bicarbonate, NaHCO3, (BDH); and 1.60g Sodium Carbonate, Na2CO3, (BDH) to 500 ml of distilled water.
   -Dissolve ingredients by mixing.
   -Adjust to a pH of 9.6 with Sodium Hydroxide.
   -Adjust solution to a final volume of 1000ml with distilled water.
4. Wash Solution- 50mM Tris, O.14 M NaCl, 0.5% Tween 20, pH 8.0
   -Add 28 ml of 5M NaCl; 50 ml 1M Tris; and 5 ml Tween 20 to a 1000 ml Erylenmeyer flask.
   -Adjust pH to 8.0.
   -Adjust solution to a final volume of 1000 ml with Distilled Water.
5. Postcoat Solution- 50mM Tris, O.15 M NaCl, 0.2% Gelatin, pH 8.0
   -Add 1.5 ml of 5M NaCl; 2.5 ml of 1M Tris; and 0.1 g of Gelatin into a 50 ml Erylenmeyer flask.

-Adjust pH to 8.0.

-Adjust solution to a final volume of 50 ml with Distilled water.

6. Sample/Conjugate Diluent- 50mM Tris, O.15 M NaCl, 0.2% Gelatin, 0.5% Tween 20, pH 8.0

-Combine 3.0 ml of5M NaCl with 5 ml 1M Tris, 0.2 g Gelatin, and 500 ul of Tween 20 in a 100 ml Erylenmeyer flask.

-Adjust pH to 8.0.

-Adjust final volume with Distilled Water (100 ml)

7. Stop Solution- 1% Sodium Dodecyl Sulfate, SDS, (AnalaR)

-Add 10 g of SDS into 1000 ml Distilled Water.

Method:

1.Dilute all standards and serum samples in Coating Buffer:
Standard- Make an initial dilution of 1 ul into 2.13 ml (10 000 ng/ml). Take 1600 ul of previous dilution and add it to 400 ul of diluent for a concentration of 8000 ng/ml. Starting at a dilution of 1:8000, prepare a 2-fold dilution series of the Bovine IgG Reference Serum.
Serum Samples-Make an initial dilution of 10 ul into 990 ul (1:100). From here make a 250-fold dilution (4 ul into 996 ul).
2.Transfer 100 ul of the standard and serum sample to predetermined wells.
3.Incubate at 37 C for 1 hour.
4.After incubation, dump Coating Solution from the plate.
6.Wash each well with 200 ul of Wash Solution. Repeat four more times.
7.Add 200ul of Postcoat Solution to each well.
8.Incubate 30 minutes at room temperature.
9.Repeat step 6.
10.Dilute the Recombinant Protein G-HRP Conjugate by pipetting 2 ul of conjugate into 10ml of diluent (1/5000).
11.Transfer 100ul of the Protein G-HRP Conjugate into each well.
12.Incubate the plate for 60 minutes at room temperature.
13.Repeat step 6.
17.Transfer 100ul of Substrate Solution to each well.
18.Incubate the plate for 12 minutes at room temperature.
19.Add 100ul of Stop Solution to each well.
20.Using a microtiter plate reader, read the O.D.'s of the wells at 405nm.

"Universal" Enzyme Linked Immunosorbent Assay (ELISA) for the Dectection of IgG in Mule/White-Tail Deer.

Equipment

1. BioRad Microplate Reader, (BioRad)
2. Pipettes-capeable of delivering 1- 10000ul, (Eppendorf, Pipettman)
3. Pipette Tips 1- 10000 ul volume, (BioRad, VWR)
4. Refrigerator (Kelvinator)

Material and Supplies:

1. ELISA Dynex Immulon IV plates, (VWR)
2. Polypropylene Culture Tubes, Polypropylene 12x75 mm and 17x100mm, (VWR)
3. ABTS (Gibco-Life Technologies)
4. Tween 20, (VWR)
5. Trisma base (Sigma)
8. Mule Deer IgG Reference Serum (Dele Ogunremi)
9. Recombinant Protein G-HRP (Zymed 1139882)
10. Sodium Chloride (BDH Inc.)
11. Gelatin, (Difco)
12. 1 % Sodium Dodecyl Sulfate

Solution Preparation:

1. 5M Sodium Chloride, (BDH)
   -Add 292.2g into 1000ml of Distilled Water.
2. 1M Tris, (Sigma)
   -Add 60.55g into 500ml of Distilled Water.
   -pH solution with 1M Hydrochloric Acid, pH 8.0, (AnalaR).
3. Coating Buffer- 0.05M Sodium carbonate, pH 9.6
   -Add 0.2g Sodium Azide, NaN3, (BDH); 2.94g Sodium Bicarbonate, NaHCO3, (BDH); and 1.60g Sodium Carbonate, Na2CO3, (BDH) to 500 ml of distilled water.
   -Dissolve ingredients by mixing.
   -Adjust to a pH of 9.6 with Sodium Hydroxide.
   -Adjust solution to a final volume of 1000ml with distilled water.
4. Wash Solution- 50mM Tris, O.14 M NaCl, 0.5% Tween 20, pH 8.0
   -Add 28 ml of 5M NaCl; 50 ml 1M Tris; and 5 ml Tween 20 to a 1000 ml Erylenmeyer flask.
   -Adjust pH to 8.0.
   -Adjust solution to a final volume of 1000 ml with Distilled Water.
5. Postcoat Solution- 50mM Tris, O.15 M NaCl, 0.2% Gelatin, pH 8.0
   -Add 1.5 ml of 5M NaCl; 2.5 ml of 1M Tris; and 0.1 g of Gelatin into a 50 ml Erylenmeyer flask.
   -Adjust pH to 8.0.
   -Adjust solution to a final volume of 50 ml with Distilled water.
6. Sample/Conjugate Diluent- 50mM Tris, O.15 M NaCl, 0.2% Gelatin, 0.5% Tween 20, pH 8.0

-Combine 3.0 ml of5M NaCl with 5 ml 1M Tris, 0.2 g Gelatin, and 500 ul of Tween 20 in a 100 ml Erylenmeyer flask.
-Adjust pH to 8.0.
-Adjust final volume with Distilled Water (100 ml)

7. Stop Solution- 1% Sodium Dodecyl Sulfate, SDS, (AnalaR)
-Add 10 g of SDS into 1000 ml Distilled Water.

Method:

1.Dilute all standards and serum samples in Coating Buffer:
Standard- Make an initial dilution of 1 ul into 667 ul. Take 160 ul of previous dilution and add it to 340 ul of diluent. From there prepare a 2-fold dilution series of the Mule Deer IgG Reference Serum.
Serum Samples-Make an initial dilution of 10 ul into 990 ul (1:100). From here make a 200-fold dilution (5 ul into 995 ul, 1:20 000).
2.Transfer 100 ul of the standard and serum sample to predetermined wells.
3.Incubate at 37 C for 1 hour.
4.After incubation, dump Coating Solution from the plate.
6.Wash each well with 200 ul of Wash Solution. Repeat four more times.
7.Add 200ul of Postcoat Solution to each well.
8.Incubate 30 minutes at room temperature.
9.Repeat step 6.
10.Dilute the Recombinant Protein G-HRP Conjugate by pipetting 2 ul of conjugate into 10ml of diluent (1/5000).
11.Transfer 100ul of the Protein G-HRP Conjugate into each well.
12.Incubate the plate for 60 minutes at room temperature.
13.Repeat step 6.
17.Transfer 100ul of Substrate Solution to each well.
18.Incubate the plate for 9 minutes at room temperature.
19.Add 100ul of Stop Solution to each well.
20.Using a microtiter plate reader, read the O.D.'s of the wells at 405nm.

"Universal" Enzyme Linked Immunosorbent Assay (ELISA) for the Detection of IgG in Elk.

Material and Supplies:

A. Equipment
1. BioRad Microplate Reader, (BioRad)
2. Pipettes-capeable of delivering 1- 10000ul, (Eppendorf, Pipettman)
3. Pipette Tips 1- 10000 ul volume, (BioRad, VWR)
4. Refrigerator (Kelvinator)

B. Material and Supplies:
1. ELISA Dynex Immulon IV plates, (VWR)
2. Polypropylene Culture Tubes, Polypropylene 12x75 mm and 17x100mm, (VWR)
3. ABTS (Gibco-Life Technologies)
4. Tween 20, (VWR)
5. Trisma base (Sigma)
8. Elk IgG Reference Serum (Dele Ogunremi)
9. Recombinant Protein G-HRP (Zymed 1139882)
10.Sodium Chloride (BDH Inc.)
11. Gelatin, (Difco)
12. 1 % Sodium Dodecyl Sulfate

C. Solution Preparation:
1. 5M Sodium Chloride, (BDH)
    -Add 292.2g into 1000ml of Distilled Water.
2. 1M Tris, (Sigma)
    -Add 60.55g into 500ml of Distilled Water.
    -pH solution with 1M Hydrochloric Acid, pH 8.0, (AnalaR).
3. Coating Buffer- 0.05M Sodium carbonate, pH 9.6
    -Add 0.2g Sodium Azide, NaN3, (BDH); 2.94g Sodium Bicarbonate, NaHCO3, (BDH); and 1.60g Sodium Carbonate, Na2CO3, (BDH) to 500 ml of distilled water.
    -Dissolve ingredients by mixing.
    -Adjust to a pH of 9.6 with Sodium Hydroxide.
    -Adjust solution to a final volume of 1000ml with distilled water.
4. Wash Solution- 50mM Tris, O.14 M NaCl, 0.5% Tween 20, pH 8.0
    -Add 28 ml of 5M NaCl; 50 ml 1M Tris; and 5 ml Tween 20 to a 1000 ml Erylenmeyer flask.
    -Adjust pH to 8.0.
    -Adjust solution to a final volume of 1000 ml with Distilled Water.
5. Postcoat Solution- 50mM Tris, O.15 M NaCl, 0.2% Gelatin, pH 8.0
    -Add 1.5 ml of 5M NaCl; 2.5 ml of 1M Tris; and 0.1 g of Gelatin into a 50 ml Erylenmeyer flask.
    -Adjust pH to 8.0.

-Adjust solution to a final volume of 50 ml with Distilled water.

6. Sample/Conjugate Diluent- 50mM Tris, O.15 M NaCl, 0.2% Gelatin, 0.5% Tween 20, pH 8.0
   -Combine 3.0 ml of5M NaCl with 5 ml 1M Tris, 0.2 g Gelatin, and 500 ul of Tween 20 in a 100 ml Erylenmeyer flask.
   -Adjust pH to 8.0.
   -Adjust final volume with Distilled Water (100 ml)
7. Stop Solution- 1% Sodium Dodecyl Sulfate, SDS, (AnalaR)
   -Add 10 g of SDS into 1000 ml Distilled Water.

Method:

1.Dilute all standards and serum samples in Coating Buffer:
Standard- Make an initial dilution of 1 ul into 920 ul. Take 160 ul of previous dilution and add it to 340 ul of diluent. From there prepare a 2-fold dilution series of the Elk IgG Reference Serum.
Serum Samples-Make an initial dilution of 10 ul into 990 ul (1:100). From here make a 200-fold dilution (5 ul into 995 ul, 1:20 000).
2.Transfer 100 ul of the standard and serum sample to predetermined wells.
3.Incubate at 37 C for 1 hour.
4.After incubation, dump Coating Solution from the plate.
6.Wash each well with 200 ul of Wash Solution. Repeat four more times.
7.Add 200ul of Postcoat Solution to each well.
8.Incubate 30 minutes at room temperature.
9.Repeat step 6.
10.Dilute the Recombinant Protein G-HRP Conjugate by pipetting 2 ul of conjugate into 10ml of diluent (1/5000).
11.Transfer 100ul of the Protein G-HRP Conjugate into each well.
12.Incubate the plate for 60 minutes at room temperature.
13.Repeat step 6.
17.Transfer 100ul of Substrate Solution to each well.
18.Incubate the plate for 9 minutes at room temperature.
19.Add 100ul of Stop Solution to each well.
20.Using a microtiter plate reader, read the O.D.'s of the wells at 405nm.

"Universal" Enzyme Linked Immunosorbent Assay (ELISA) for the Dectection of IgG in Bison.

Material and Supplies:

A. Equipment

1. BioRad Microplate Reader, (BioRad)
2. Pipettes-capeable of delivering 1- 10000ul, (Eppendorf, Pipettman)
3. Pipette Tips 1- 10000 ul volume, (BioRad, VWR)
4. Refrigerator (Kelvinator)

B. Material and Supplies:

1. ELISA Dynex Immulon IV plates, (VWR)
2. Polypropylene Culture Tubes, Polypropylene 12x75 mm and 17x100mm, (VWR)
3. ABTS (Gibco-Life Technologies)
4. Tween 20, (VWR)
5. Trisma base (Sigma)
8. Bison IgG Reference Serum (Dele Ogunremi)
9. Recombinant Protein G-HRP (Zymed 1139882)
10. Sodium Chloride (BDH Inc.)
11. Gelatin, (Difco)
12. 1 % Sodium Dodecyl Sulfate

C. Solution Preparation:

1. 5M Sodium Chloride, (BDH)
   -Add 292.2g into 1000ml of Distilled Water.
2. 1M Tris, (Sigma)
   -Add 60.55g into 500ml of Distilled Water.
   -pH solution with 1M Hydrochloric Acid, pH 8.0, (AnalaR).
3. Coating Buffer- 0.05M Sodium carbonate, pH 9.6
   -Add 0.2g Sodium Azide, NaN3, (BDH); 2.94g Sodium Bicarbonate, NaHCO3, (BDH); and 1.60g Sodium Carbonate, Na2CO3, (BDH) to 500 ml of distilled water.
   -Dissolve ingredients by mixing.
   -Adjust to a pH of 9.6 with Sodium Hydroxide.
   -Adjust solution to a final volume of 1000ml with distilled water.
4. Wash Solution- 50mM Tris, O.14 M NaCl, 0.5% Tween 20, pH 8.0
   -Add 28 ml of 5M NaCl; 50 ml 1M Tris; and 5 ml Tween 20 to a 1000 ml Erylenmeyer flask.
   -Adjust pH to 8.0.
   -Adjust solution to a final volume of 1000 ml with Distilled Water.
5. Postcoat Solution- 50mM Tris, O.15 M NaCl, 0.2% Gelatin, pH 8.0
   -Add 1.5 ml of 5M NaCl; 2.5 ml of 1M Tris; and 0.1 g of Gelatin into a 50 ml Erylenmeyer flask.
   -Adjust pH to 8.0.

-Adjust solution to a final volume of 50 ml with Distilled water.

6. Sample/Conjugate Diluent- 50mM Tris, O.15 M NaCl, 0.2% Gelatin, 0.5% Tween 20, pH 8.0
   -Combine 3.0 ml of5M NaCl with 5 ml 1M Tris, 0.2 g Gelatin, and 500 ul of Tween 20 in a 100 ml Erylenmeyer flask.
   -Adjust pH to 8.0.
   -Adjust final volume with Distilled Water (100 ml)
7. Stop Solution- 1% Sodium Dodecyl Sulfate, SDS, (AnalaR)
   -Add 10 g of SDS into 1000 ml Distilled Water.

Method:

1.Dilute all standards and serum samples in Coating Buffer:
Standard- Make an initial dilution of 3 ul into 177 ul. Take 160 ul of previous dilution and add it to 340 ul of diluent. From there prepare a 2-fold dilution series of the Elk IgG Reference Serum.
Serum Samples-No serum samples available for testing.
2.Transfer 100 ul of the standard and serum sample to predetermined wells.
3.Incubate at 37 C for 1 hour.
4.After incubation, dump Coating Solution from the plate.
6.Wash each well with 200 ul of Wash Solution. Repeat four more times.
7.Add 200ul of Postcoat Solution to each well.
8.Incubate 30 minutes at room temperature.
9.Repeat step 6.
10.Dilute the Recombinant Protein G-HRP Conjugate by pipetting 2 ul of conjugate into 10ml of diluent (1/5000).
11.Transfer 100ul of the Protein G-HRP Conjugate into each well.
12.Incubate the plate for 60 minutes at room temperature.
13.Repeat step 6.
17.Transfer 100ul of Substrate Solution to each well.
18.Incubate the plate for 9 minutes at room temperature.
19.Add 100ul of Stop Solution to each well.
20.Using a microtiter plate reader, read the O.D.'s of the wells at 405nm.

"Universal" Enzyme Linked Immunosorbent Assay (ELISA) for the Dectection of IgG in Llama.

Material and Supplies:

A. Equipment
1. BioRad Microplate Reader, (BioRad)
2. Pipettes-capeable of delivering 1- 10000ul, (Eppendorf, Pipettman)
3. Pipette Tips 1- 10000 ul volume, (BioRad, VWR)
4. Refrigerator (Kelvinator)

B. Material and Supplies:
1. ELISA Dynex Immulon IV plates, (VWR)
2. Polypropylene Culture Tubes, Polypropylene 12x75 mm and 17x100mm, (VWR)
3. ABTS (Gibco-Life Technologies)
4. Tween 20, (VWR)
5. Trisma base (Sigma)
6. Recombinant Protein G-HRP (Zymed 1139882)
7.Sodium Chloride (BDH Inc.)
8. Gelatin, (Difco)
9. 1 % Sodium Dodecyl Sulfate

C. Solution Preparation:
1. 5M Sodium Chloride, (BDH)
   -Add 292.2g into 1000ml of Distilled Water.
2. 1M Tris, (Sigma)
   -Add 60.55g into 500ml of Distilled Water.
   -pH solution with 1M Hydrochloric Acid, pH 8.0, (AnalaR).
3. Coating Buffer- 0.05M Sodium carbonate, pH 9.6
   -Add 0.2g Sodium Azide, NaN3, (BDH); 2.94g Sodium Bicarbonate, NaHCO3, (BDH); and 1.60g Sodium Carbonate, Na2CO3, (BDH) to 500 ml of distilled water.
   -Dissolve ingredients by mixing.
   -Adjust to a pH of 9.6 with Sodium Hydroxide.
   -Adjust solution to a final volume of 1000ml with distilled water.
4. Wash Solution- 50mM Tris, O.14 M NaCl, 0.5% Tween 20, pH 8.0
   -Add 28 ml of 5M NaCl; 50 ml 1M Tris; and 5 ml Tween 20 to a 1000 ml Erylenmeyer flask.
   -Adjust pH to 8.0.
   -Adjust solution to a final volume of 1000 ml with Distilled Water.
5. Postcoat Solution- 50mM Tris, O.15 M NaCl, 0.2% Gelatin, pH 8.0
   -Add 1.5 ml of 5M NaCl; 2.5 ml of 1M Tris; and 0.1 g of Gelatin into a 50 ml Erylenmeyer flask.
   -Adjust pH to 8.0.
   -Adjust solution to a final volume of 50 ml with Distilled water.

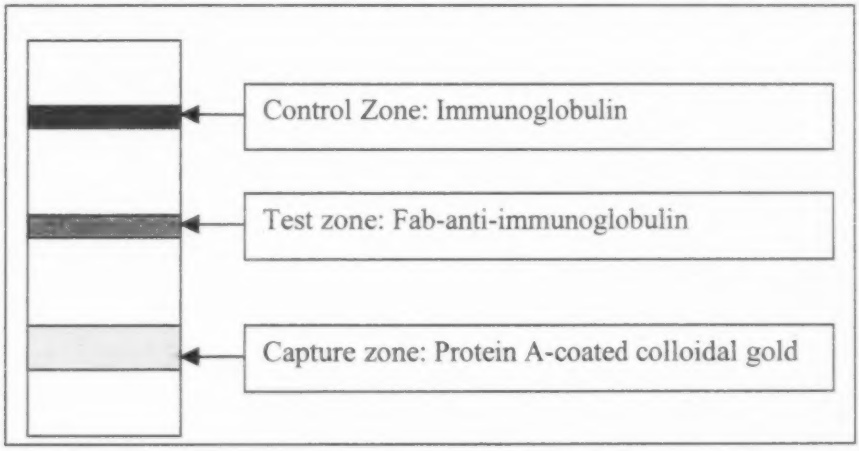
6. Sample/Conjugate Diluent- 50mM Tris, O.15 M NaCl, 0.2% Gelatin, 0.5% Tween 20, pH 8.0
   -Combine 3.0 ml of5M NaCl with 5 ml 1M Tris, 0.2 g Gelatin, and 500 ul of Tween 20 in a 100 ml Erylenmeyer flask.
   -Adjust pH to 8.0.
   -Adjust final volume with Distilled Water (100 ml)
7. Stop Solution- 1% Sodium Dodecyl Sulfate, SDS, (AnalaR)
   -Add 10 g of SDS into 1000 ml Distilled Water.

Method:

1.Dilute all standards and serum samples in Coating Buffer:
Standard- No standards available for testing.
Serum Samples-Make an initial dilution of 10 ul into 990 ul (1:100). From here make a 100-fold dilution (10 ul into 990 ul, 1:10 000).
2.Transfer 100 ul of the standard and serum sample to predetermined wells.
3.Incubate at 37 C for 1 hour.
4.After incubation, dump Coating Solution from the plate.
6.Wash each well with 200 ul of Wash Solution. Repeat four more times.
7.Add 200ul of Postcoat Solution to each well.
8.Incubate 30 minutes at room temperature.
9.Repeat step 6.
10.Dilute the Recombinant Protein G-HRP Conjugate by pipetting 2 ul of conjugate into 10ml of diluent (1/5000).
11.Transfer 100ul of the Protein G-HRP Conjugate into each well.
12.Incubate the plate for 60 minutes at room temperature.
13.Repeat step 6.
17.Transfer 100ul of Substrate Solution to each well.
18.Incubate the plate for 9 minutes at room temperature.
19.Add 100ul of Stop Solution to each well.
20.Using a microtiter plate reader, read the O.D.'s of the wells at 405nm.

Appendix F. Diagram of the prototype immuno-chromatographic assay for IgG detection

Schematic representation of paper strip and zones for immuno-cromatography



Schematic representation of assay steps